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Pharmacological Characterization of the Receptor Mediating the Anorexigenic Action of the Octadecaneuropeptide: Evidence for an Endozepinergic Tone Regulating Food Intake

Jean-Claude do Rego*[,1], Marie-Hélène Orta[1], Jérôme Leprince[2], Marie-Christine Tonon[2], Hubert Vaudry[2] and Jean Costentin[1]

[1]CNRS FRE 2735, Laboratory of Experimental Neuropsychopharmacology, European Institute for Peptide Research (IFRMP 23), Faculty of Medicine and Pharmacy, University of Rouen, Rouen Cedex, France; [2]Inserm U413, Laboratory of Cellular and Molecular Neuroendocrinology, European Institute for Peptide Research (IFRMP 23), University of Rouen, Mont-Saint-Aignan, France

Peptides of the endozepine family, including diazepam-binding inhibitor, the triakontatetraneuropeptide, and the octadecaneuropeptide (ODN), act through three types of receptors, that is, central-type benzodiazepine receptors (CBR), peripheral-type (mitochondrial) benzodiazepine receptors (PBR) and a metabotropic receptor positively coupled to phospholipase C via a pertussis toxin-sensitive G protein. We have previously reported that ODN exerts a potent anorexigenic effect in rat and we have found that the action of ODN is not affected by the mixed CBR/PBR agonist diazepam. In the present report, we have tested the possible involvement of the metabotropic receptor in the anorexigenic activity of ODN. Intracerebroventricular administration of the C-terminal octapeptide (OP) and its head-to-tail cyclic analog cyclo$_{1-8}$OP (cOP) at a dose of 100 ng mimicked the inhibitory effect of ODN on food intake in food-deprived mice. The specific CBR antagonist flumazenil and the PBR antagonist PK11195 did not prevent the effect of ODN, OP, and cOP on food consumption. In contrast, the selective metabotropic endozepine receptor antagonist cyclo$_{1-8}$[DLeu^5]OP (100–1000 ng; cDLOP) suppressed the anorexigenic effect of ODN, OP, and cOP. At the highest concentration tested (1000 ng), cDLOP provoked by itself a significant increase in food intake. Taken together, the present results indicate that the anorexigenic effect of ODN and OP is mediated through activation of the metabotropic receptor recently characterized in astrocytes. The data also suggest that endogenous ODN, acting via this receptor, exerts an inhibitory tone on feeding behavior.
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Keywords: endozepines; diazepam-binding inhibitor; octadecaneuropeptide; feeding behavior; metabotropic receptors; mouse

INTRODUCTION

The term endozepines designates a family of regulatory neuropeptides that have been originally isolated from rat brain extracts on the basis of their ability to displace benzodiazepines from their binding sites (Guidotti *et al*, 1983; Tonon *et al*, 2006). All endozepines characterized so far derive from diazepam-binding inhibitor (DBI), an 86 amino-acid polypeptide precursor which has the potential to generate several biologically active fragments including the triakontatetraneuropeptide DBI$_{17-50}$ (TTN) (Slobodyansky *et al*, 1989) and the octadecaneuropeptide DBI$_{33-50}$ (ODN) (Ferrero *et al*, 1986). The DBI gene is widely expressed in the central nervous system (Alho *et al*, 1988; Tong *et al*, 1991; Yanase *et al*, 2002). In particular, high concentrations of endozepines have been found in brain areas which play a major role in the control of feeding behavior such as the arcute nucleus, the dorso-medial and ventro-medial hypothalamic nuclei, and the lateral hypothalamic area (Alho *et al*, 1985; Malagon *et al*, 1993; Tonon *et al*, 1990).

Intracerebroventricular (i.c.v.) injection of low doses of ODN causes a marked reduction of food consumption in both food deprived and normally fed rodents (Garcia de Mateos-Verchere *et al*, 2001). The anorexigenic effect of ODN is long lasting, does not give rise to rapid tolerance, and is associated with a substantial loss of weight (Garcia de Mateos-Verchere *et al*, 2001). In rats deprived of food for 12 h, i.c.v. administration of ODN reduces the expression of neuropeptide Y (NPY), an orexigenic neuropeptide, and activates the expression of proopiomelanocortin, the precursor of the anorexigenic peptide α-melanocyte-stimulating hormone (Compère *et al*, 2003, 2005), suggesting that

*Correspondence: Dr J-C do Rego, CNRS FRE 2735, Laboratory of Experimental Neuropsychopharmacology, European Institute for Peptide Research (IFRMP 23), Faculty of Medicine and Pharmacy, University of Rouen, 22 Boulevard Gambetta, 76183 Rouen Cedex, France, Tel: +33 2 35148602, Fax: +33 2 35148603,
E-mail: jean-claude.dorego@univ-rouen.fr
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NPY- and POMC-producing neurons of the arcute nucleus are involved in the anorexigenic effect of ODN.

The biological effects of endozepines are mediated through three types of receptors. DBI and ODN act as inverse agonists of central-type benzodiazepine receptors (CBR) associated with the $GABA_A$ receptor complex (Guidotti *et al*, 1983; Tonon *et al*, 1989; Bormann, 1991; Louiset *et al*, 1993). TTN acts preferentially through peripheral-type benzodiazepine receptors (PBR) located either at the outer mitochondrial membrane or at the plasma membrane level (Guidotti *et al*, 1989; Berkovich *et al*, 1990; Gandolfo *et al*, 2001). Finally, ODN can also activate a metabotropic receptor positively coupled to phospholipase C through a pertussis toxin-sensitive G-protein (Patte *et al*, 1995; Gandolfo *et al*, 1997). We have previously shown that the anorexigenic effect of ODN was not affected by the mixed CBR/PBR agonist diazepam (Garcia de Mateos-Verchere *et al*, 2001). The aim of the present study was to investigate the possible involvement of the metabotropic receptor in the action of endozepines on feeding behavior.

MATERIALS AND METHODS

Animals

Male Swiss albinos CD1 mice (Iffa-Credo/Charles River, Saint-Germain sur l'Arbresle, France), weighing 22–25 g, were housed 20 in Makrolon cages (L: 40 cm, W: 25 cm, and H: 18 cm), with free access to standard semisynthetic laboratory diet (U.A.R., Villemoisson-sur-Orge, France) and tap water. The animals were kept in a ventilated room at a temperature of $22 \pm 1^{\circ}C$ under a 12-h light/12-h dark cycle (light on between 0700 and 1900).

All the experiments were carried out between 0900 and 1800 in testing rooms adjacent to the animal rooms. Animal manipulations were performed according to the European Community Council Directive of 24 November 1986 (86:609:EEC), were approved by the local Ethical Committee (authorization numbers: N/10-04-04-12 and N/13-04-04-15) and were conducted by authorized investigators. Each animal was used once and then immediately killed.

Drugs and Solutions

The metabotropic endozepine receptor agonists ODN (Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys), octapeptide (OP) (Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys) and $cyclo_{1-8}$OP (Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys; cOP), and the metabotropic endozepine receptor antagonist $cyclo_{1-8}[D\text{Leu}^5]$OP (Arg-Pro-Gly-Leu-DLeu-Asp-Leu-Lys; cDLOP) were synthesized by solid phase methodology as described previously (Leprince *et al*, 1998, 2001). The peptides were dissolved in saline (0.9% NaCl). Flumazenil (Ro 15–1788) and PK11195 (Sigma Aldrich, Saint-Quentin Fallavier, France) were dissolved in 2.5% dimethylsulfoxide in saline. For PK11195, 0.5% Tween-80 was added to facilitate solubilization. All solutions were prepared just before injections.

I.c.v. Injection

Free-hand i.c.v. injections (10 μl/mouse) of saline, peptides and/or drugs were made in the left ventricle according to the procedure of Haley and McCormick (1957), using a microsyringe (50 μl; Hamilton, Bonaduz, Switzerland) connected to a needle (diameter 0.5 mm), of which the bevel protruded only 3.5 mm from a guard limiting its penetration into the brain. The injection in immobilized mice lasted approximately 5 s. I.c.v. injections were performed by an experienced investigator, who frequently controlled the regularity and success of the injections, using methylene blue dye and who observed (after killing and frontal brain sectioning) that the injection was successful in more than 95% of the trials. The i.c.v. injection method was approved by the Regional Ethical Committee for Animal Experimentation (Normandy; no. N/10-04-04-12).

Food Consumption Experiments in Food-Deprived Mice or Food-Restricted Mice

Two days before the experiments, mice were isolated in individual cages (L: 24 cm, W: 10, and H: 7 cm) with free access to water and the pellets of food laid down the floor of the cages, in order to make the animals accustomed to the test conditions. For experiments with food-deprived mice (fasted mice) (in order to evaluate an anorexigenic effect), 18 h before testing (1500–0900 h), animals were totally deprived of food and had access to tap water *ad libitum*. For experiments with food-restricted mice (in order to evaluate an orexigenic effect), 18 h before testing (1500–0900 h), animals had access to only 3 g of food (that represented approximately half of their daily consumption) and water *ad libitum*. In all groups, mice had access to a weighed food pellet (5 g) deposited on the floor of the cage 10 min after i.c.v. administration of saline, peptides, and/or drugs. Thereafter, the pellet was briefly (<20 s) removed with forceps and weighed every 30 min, for 3 h. The food consumption method was approved by the Regional Ethical Committee for Animal Experimentation (Normandy; no. N/13-04-04-15).

Measurement of Locomotor Activity

Ten min after i.c.v. administration of saline or peptides, the animals were placed individually in $20 \times 20 \times 30$ cm compartments, in a lighted and quiet room. Locomotor activity was assessed automatically in a Digiscan actimeter (Omnitech Electronics Inc, Colombus, OH), which monitored the horizontal displacements and vertical movements, including rearing, leaning, and jumping. The responses were expressed as the total number of beams crossed by mice during four consecutive 15-min periods.

Statistical Analysis

Data are expressed as means $\pm$ SEM. Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by a *post hoc* multiple comparison Student–Newman–Keuls test. Antagonistic effects were analyzed using two-way ANOVA, and a *post hoc* multiple comparison Student–Newman–Keuls test was used for multiple comparisons between groups. A probability level of 0.05 or lower was considered as statistically significant.

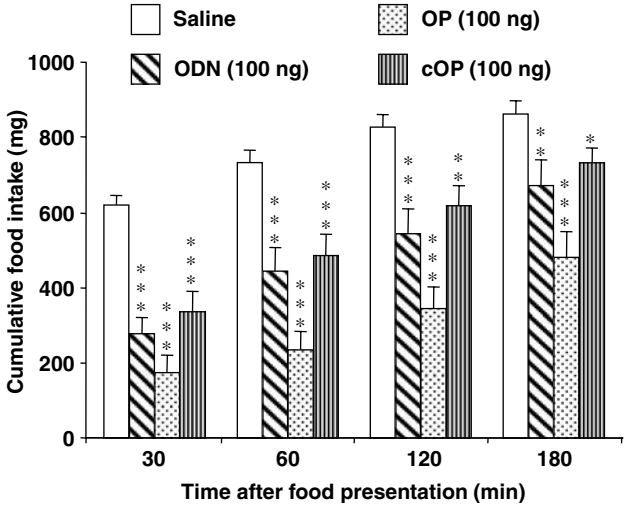

Figure 1 Time course of the effect of ODN, OP, or cOP on food intake in food-deprived mice. Mice deprived of food for 18 h were injected i.c.v. (10 µl) with saline, ODN (100 ng/mouse), OP (100 ng/mouse), or cOP(100 ng/mouse). Ten min after i.c.v. injection, each animal had access to a weighed food pellet. Cumulative food intake was measured during 3 h at the time indicated. Mean ± SEM represents data from 14 mice per group. Student–Newman–Keuls *post hoc* test: *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs* saline-injected mice.

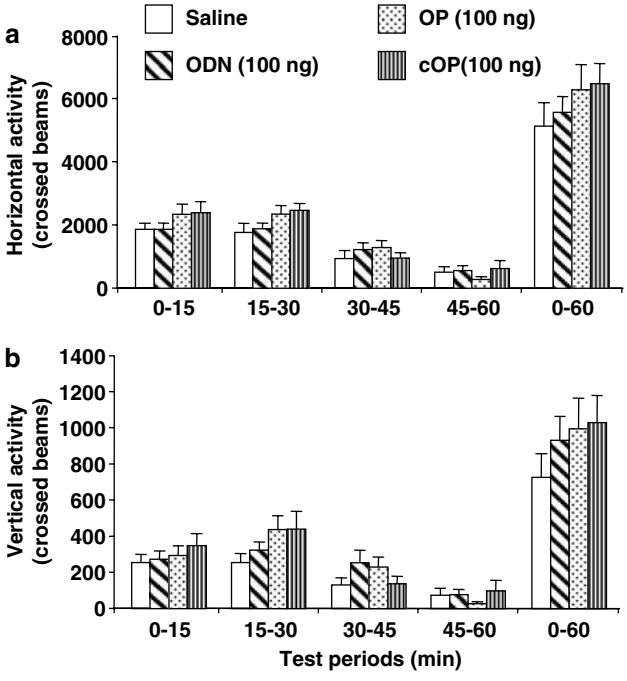

Figure 2 Effect of ODN, OP, or cOP on locomotor activity. Mice deprived of food for 18 h were injected i.c.v. (10 µl) with saline, ODN (100 ng/mouse), OP (100 ng/mouse), or cOP (100 ng/mouse). Ten min after i.c.v. injection, animals were introduced into the actimeters. The horizontal (a) and vertical (b) components of motor activity were measured during four consecutive periods of 10 min. Mean ± SEM represents data from 12 mice per group.

RESULTS

Effects of ODN, OP, and cOP on Food Intake in Food-Deprived Mice

In food-deprived mice, i.c.v. injection of 100 ng ODN, OP, or cOP significantly reduced ($p<0.001$) food intake during the first 30-min period of testing. The mean ± SEM percentage of food intake inhibition induced by these peptides is: 55.3 ± 6.8% for ODN, 68.9 ± 8.1% for OP, and 51.5 ± 7.4% for cOP. Thereafter, the anorexigenic effect of all three peptides gradually vanished but the cumulative food consumption remained significantly reduced during the whole 3-h test period (Figure 1).

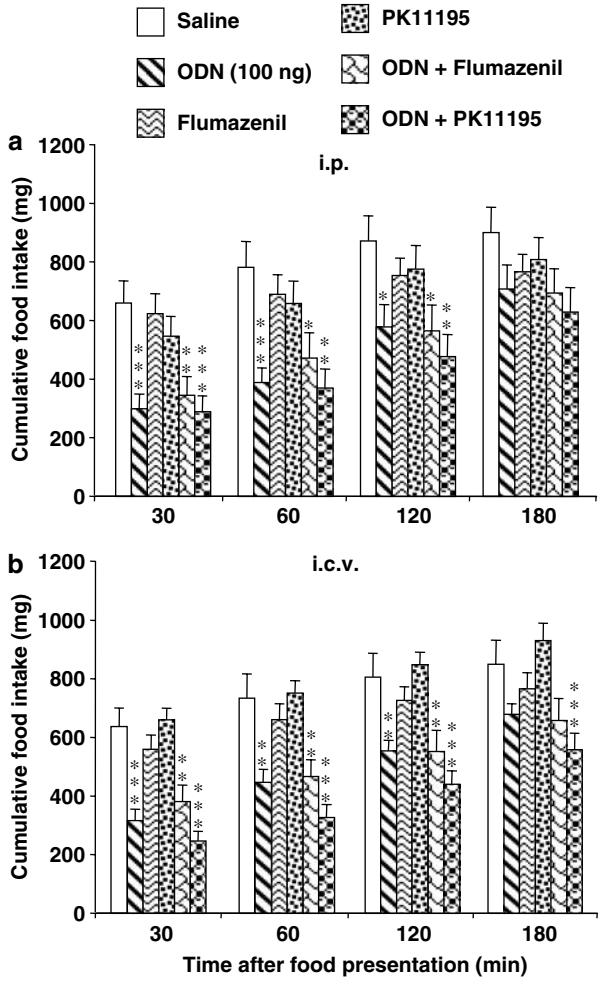

Figure 3 Effect of flumazenil and PK11195 on ODN-induced inhibition of food intake in food-deprived mice. (a) Mice deprived of food for 18 h were injected i.p. with saline, flumazenil (2 mg/kg), or PK11195 (2 mg/kg), 30 min before i.c.v. injection (10 µl) of saline, or ODN (100 ng/mouse). (b) Mice deprived of food for 18 h were injected i.c.v. (10 µl) with saline, ODN (100 ng/mouse), flumazenil (2 µg/mouse), PK11195 (2 µg/mouse), or with ODN plus flumazenil or PK11195. Ten min after i.c.v. injection, each animal had access to a weighed food pellet. Cumulative food intake was determined during 3 h at the time indicated. Mean ± SEM represents data from 14 mice per group. Student–Newman–Keuls *post hoc* test: *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs* saline-injected mice.

Effects of ODN, OP, and cOP on Locomotor Activity

The influence of i.c.v. administration of ODN, OP, or cOP on locomotor activity was measured over four consecutive periods of 15 min each (Figure 2). The peptides did not significantly modify horizontal (Figure 2a) and vertical (Figure 2b) locomotor activities at any time of the test period.

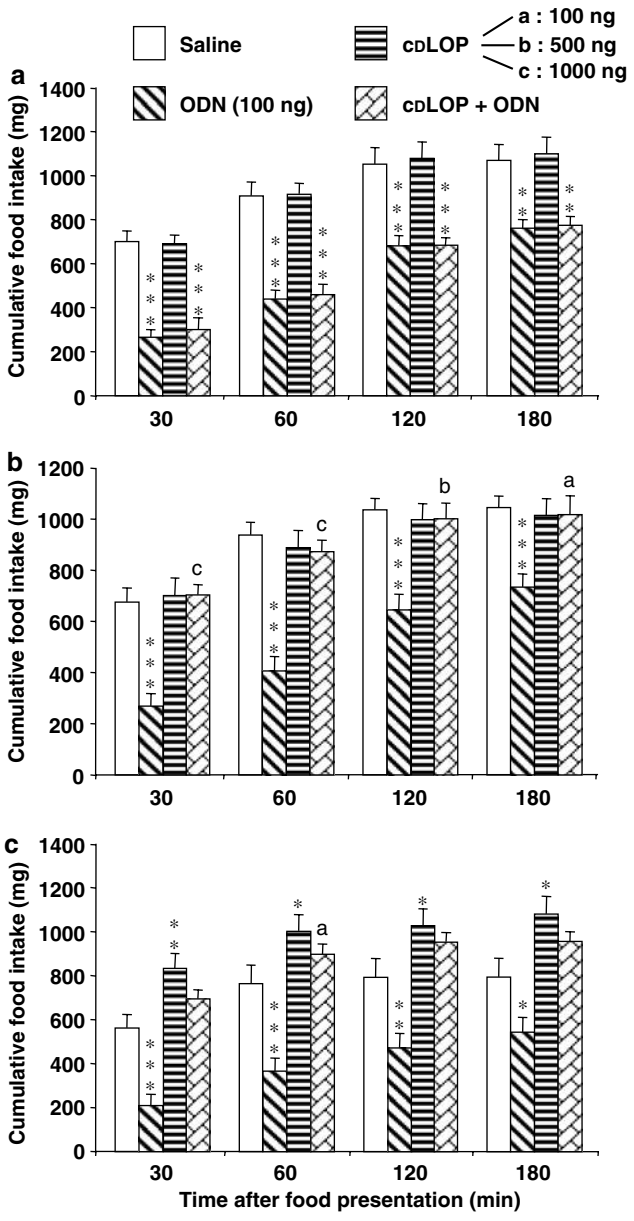

Figure 4 Effect of graded doses of cDLOP on ODN-induced inhibition of food intake in food-deprived mice. Mice deprived of food for 18 h, were injected i.c.v. (10 µl) with saline, or ODN (100 ng/mouse) in the absence or presence of increasing doses of cyclo$_{1-8}$[DLeu^5]OP (cDLOP; a, 100 ng/mouse; b, 500 ng/mouse; c, 1000 ng/mouse). Ten min after i.c.v. injection, each animal had access to a weighed food pellet. Cumulative food intake was measured during 3 h at the time indicated. Mean ± SEM represents data from 10 to 24 mice per group. Student–Newman–Keuls test: $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs* saline-injected mice. A two-way ANOVA revealed a significant interaction: between cDLOP (500 ng/mouse) and ODN at 30 min $F(1,36)=14.6$, $^cp<0.001$, at 60 min $F(1,36)=22.3$, $^cp<0.001$, at 120 min $F(1,36)=11.9$, $^bp<0.01$, and at 180 min $F(1,36)=7.2$, $^ap<0.05$; and between cDLOP (1000 ng/mouse) and ODN at 60 min after food presentation $F(1.52)=4.8$, $^ap<0.05$.

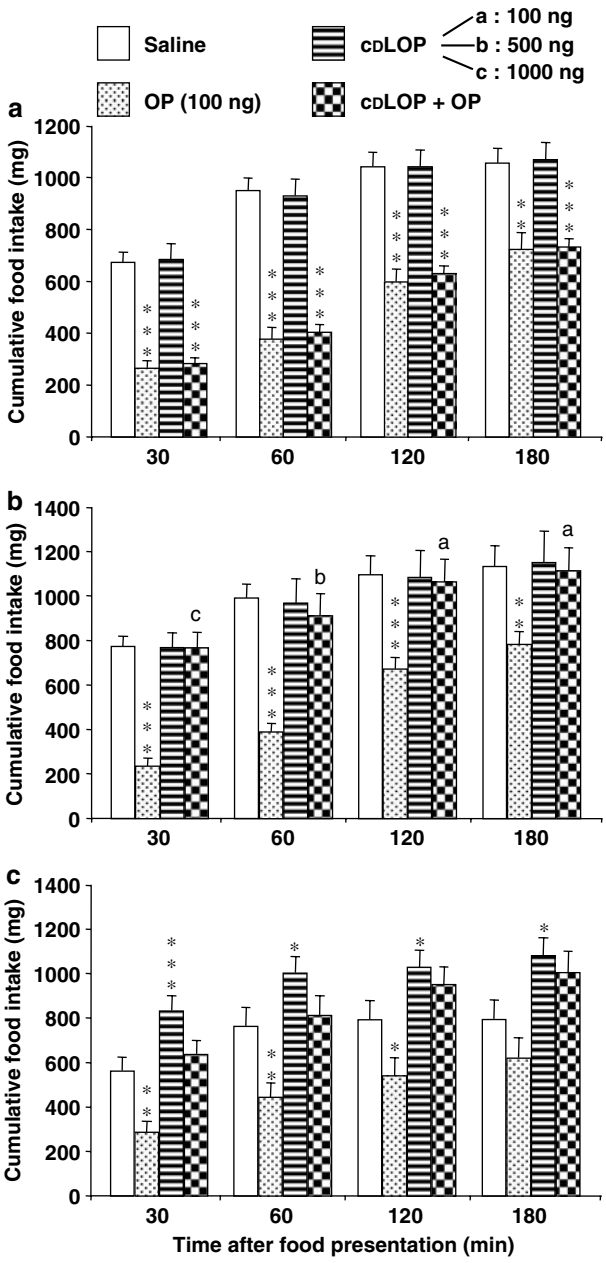

Figure 5 Effect of graded doses of cDLOP on OP-induced inhibition of food intake in food-deprived mice. Mice deprived of food for 18 h, were injected i.c.v. (10 µl) with saline, or OP (100 ng/mouse) in the absence or presence of increasing doses of cyclo$_{1-8}$[DLeu^5]OP (cDLOP; a, 100 ng/mouse; b, 500 ng/mouse; c, 1000 ng/mouse). Ten min after i.c.v. injection, each animal had access to a weighed food pellet. Cumulative food intake was measured during 3 h at the time indicated. Mean ± SEM represents data from 10 to 24 mice per group. Student–Newman–Keuls test: $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs* saline injected-mice. A two-way ANOVA revealed a significant interaction between cDLOP (500 ng/mouse) and OP at 30 min $F(1,44)=23.3$, $^cp<0.001$, at 60 min $F(1,44)=10.9$, $^bp<0.01$, at 120 min $F(1,44)=4.4$, $^ap<0.05$, and at 180 min $F(1,44)=4.2$, $^ap<0.05$.

Characterization of the Receptor Involved in the Anorexigenic Effects of ODN, OP, and cOP

To characterize the pharmacological profile of the receptor involved in the anorexigenic effect of ODN, food-deprived mice were treated with the CBR antagonist flumazenil, the PBR antagonist PK11195, or the metabotropic receptor antagonist cDLOP. Intraperitoneal (i.p.) and i.c.v. administration of flumazenil or PK11195 alone did not modify spontaneous food consumption (Figure 3a and b). Similarly, i.p. injection of flumazenil or PK11195, 30 min

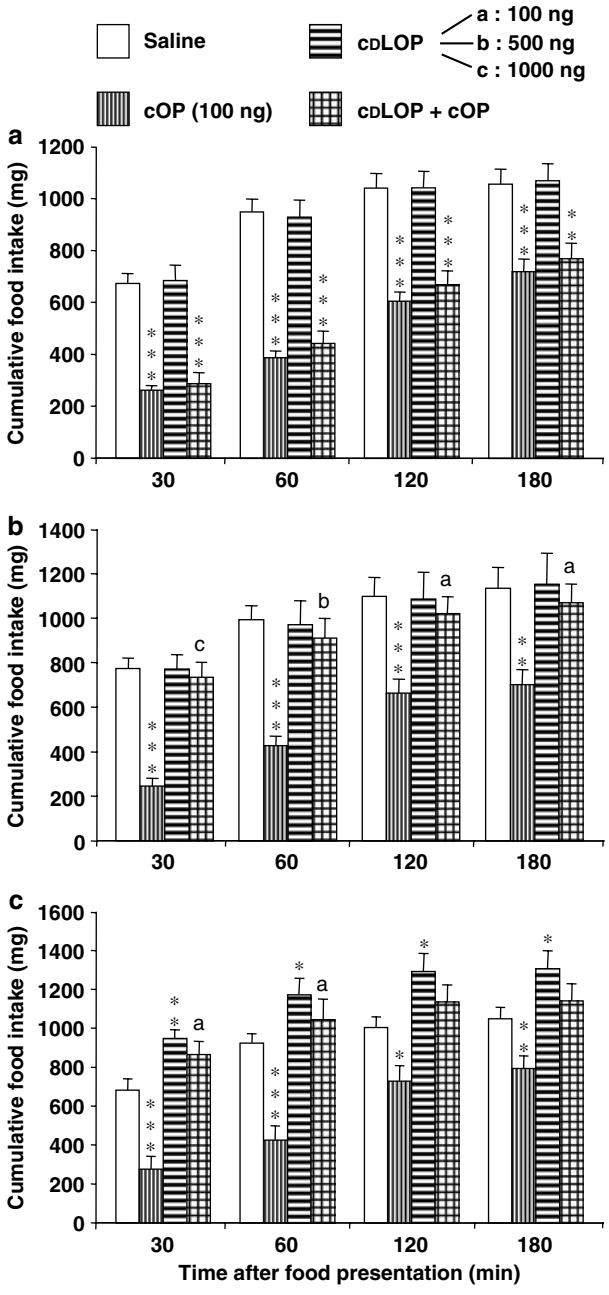

Figure 6 Effect of graded doses of cDLOP on cOP-induced inhibition of food intake in food-deprived mice. Mice deprived of food for 18 h, were injected i.c.v. (10 μl) with saline or cOP (100 ng/mouse) in the absence or presence of increasing doses of cyclo$_{1-8}$[DLeu^5]OP (cDLOP; a, 100 ng/mouse; b, 500 ng/mouse; c, 1000 ng/mouse). Ten min after i.c.v. injection, each animal had access to a weighed food pellet. Cumulative food intake was determined during 3 h at the time indicated. Mean ± SEM represents data from 10 to 24 mice per group. Student–Newman–Keuls test $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs* saline-injected mice. A two-way ANOVA revealed a significant interaction: between cDLOP (500 ng/mouse) and cOP at 30 min $F(1,44)=19.5$, $^cp<0.001$, at 60 min $F(1,44)=10.2$, $^bp<0.01$, at 120 min $F(1,44)=4.1$, $^ap<0.05$, and at 180 min $F(1,44)=4.1$, $^ap<0.05$ after food presentation; and between cDLOP (1000 ng/mouse) and ODN at 30 and 60 min after food presentation $F(1.36)=7.4$, $^ap<0.05$ and $F(1,36)=5.5$, $^ap<0.05$, respectively.

before i.c.v. administration of ODN, had no effect on ODN-induced inhibition of food intake during the 3-h test period (Figure 3a). The inhibitory effect of ODN was not affected either by i.c.v. co-administration of flumazenil or PK11195 (Figure 3b). At the lowest dose tested (100 ng/mouse, i.c.v.), cDLOP did not impair the anorexigenic effect of ODN (Figure 4a), OP (Figure 5a) and cOP (Figure 6a) in food-deprived mice. At higher doses (500 and 1000 ng/mouse, i.c.v.) cDLOP totally suppressed the inhibitory effect of ODN (Figure 4b and c), OP (Figure 5b and c) and cOP (Figure 6b and c) on food intake. At the highest dose tested (1000 ng/mouse, i.c.v.), cDLOP significantly increased food consumption in food-deprived mice, throughout the 3-h observation period (Figures 4c, 5c and 6c).

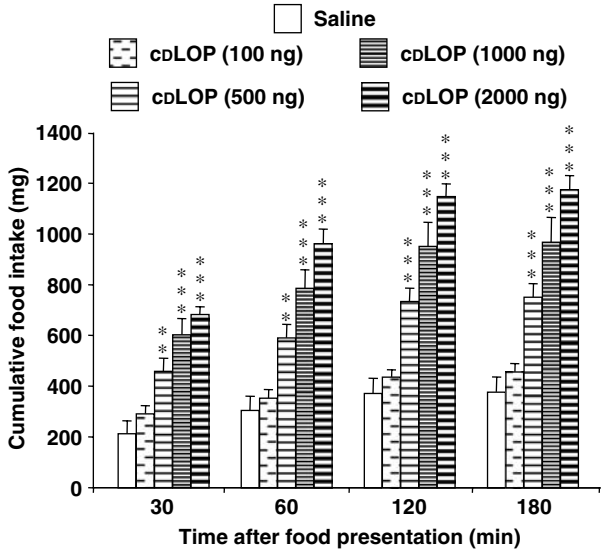

Figure 7 Effect of graded doses of cDLOP on food intake in food-restricted mice. Mice were food restricted (half of their daily food consumption) for 18 h and then injected i.c.v. (10 μl) with saline or increasing doses of cyclo$_{1-8}$[DLeu^5]OP (cDLOP; 100–2000 ng/mouse). Ten min after i.c.v. injection, each animal had access to a weighed food pellet. Cumulative food intake was determined during 3 h at the time indicated. Mean ± SEM represents data from 10 mice per group. Student–Newman–Keuls test: $^{**}p<0.01$, $^{***}p<0.001$ *vs* saline-injected mice.

Effect of cDLOP on Food Intake in Food-Restricted Mice

In mice that received approximately half of their daily consumption of food for 18 h (1500–0900), i.c.v. administration of graded doses of cDLOP (100–2000 ng/mouse) provoked a dose-related increase of cumulative food intake reaching statistical significance at a dose of 500 ng/mouse (Figure 7). For doses ranging from 500 to 2000 ng/mouse, cDLOP induced a significant orexigenic effect during the whole duration of the test with an increase in cumulative food consumption of 98.1 ± 13.8% for the 500 ng dose, 155.1 ± 25.6% for the 1000 ng, and 209.2 ± 14.6 for the 2000 ng dose, at the end of the 3-h test period (Figure 7).

Effects of Flumazenil, PK11195, and ODN on cDLOP-Induced Orexigenic Effect in Food-Restricted Mice

I.c.v. administration of flumazenil (2 μg/mouse) or PK11195 (2 μg/mouse) in food-restricted mice had no effect on food

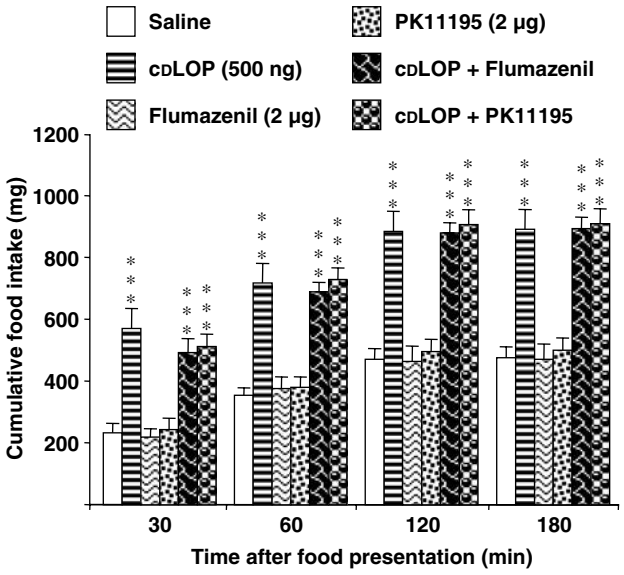

Figure 8 Effect of flumazenil or PK11195 on cDLOP-induced increase of food intake in food-restricted mice. Mice were food restricted (half of their daily food consumption) for 18 h and then injected i.c.v. (10 µl) with saline, cyclo$_{1-8}$[DLeu^5]OP (cDLOP; 500 ng/mouse), flumazenil (2 µg/mouse), PK11195 (2 µg/mouse) or with cDLOP plus flumazenil or PK11195. Ten min after i.c.v. injection, each animal had access to a weighed food pellet. Cumulative food intake was measured during 3 h at the time indicated. Mean ± SEM represents data from 12 mice per group. Student–Newman–Keuls test: ***$p < 0.001$ *vs* saline-injected mice.

consumption and did not significantly alter the orexigenic effect of cDLOP (500 ng/mouse), during the whole 3-h test period (Figure 8). In contrast, i.c.v. co-administration of ODN (10, 100, 200, or 300 ng/mouse) with cDLOP (500 ng/mouse) reversed the orexigenic effect of cDLOP and dose dependently restored its own anorexigenic effect (Figure 9). When injected alone, increasing doses of ODN (100, 200, and 300 ng/mouse) dose dependently and significantly decreased ($p < 0.05$–0.001) the cumulative food intake in food-restricted mice (Figure 9).

DISCUSSION

It has previously been reported that very low doses of ODN inhibit food intake in rat and mice (Garcia de Mateos-Verchere *et al*, 2001) but the pharmacological profile of the receptor involved in the effect of ODN had not been characterized. In the present study, we show that the anorexigenic action of ODN is mimicked by the C-terminal fragment of ODN (OP) and its head-to-tail cyclic analog (cOP). Indeed, the C-terminal domain of ODN is capable of inducing several of the effects of ODN such as the anxiogenic activity (Garcia de Mateos-Verchere *et al*, 1998a), the inhibition of apomorphine-induced yawning (Garcia de Mateos-Verchere *et al*, 1998b) and the inhibition of pentylenetetrazol-induced convulsions (Garcia de Mateos-Verchere *et al*, 1999). Interestingly, in the present study, we did not observe any effect of ODN, OP, or cOP on motor activity, indicating that the inhibitory effect of ODN and related peptides on food consumption could not be ascribed to a sedation or psychostimulant activity of these compounds.

It is now clearly established that ODN can interact with CBR associated with the $GABA_A$ receptor (Ferrero *et al*, 1986; Tonon *et al*, 1989; Slobodyansky *et al*, 1990). In addition, it has been shown that several of the effects induced by ODN are mediated via a G-protein-coupled receptor. In particular, *in vitro* studies have shown that ODN increases intracellular calcium concentration in cultured rat astrocytes through activation of a metabotropic receptor positively coupled to phospholipase C (Patte *et al*, 1995; Gandolfo *et al*, 1997). We have previously observed that the inhibitory effect of ODN on feeding behavior is not affected by diazepam, a mixed CBR and PBR agonist (Garcia de Mateos-Verchere *et al*, 2001). In order to further characterize the type of receptor mediating the anorexigenic action of ODN and its analogs, in the present study we have investigated the effect of selective endozepine receptor antagonists on ODN, OP, and cOP-induced food consumption. The specific CBR antagonist flumazenil (Bonetti *et al*, 1982) and the specific PBR antagonist PK11195 (Le Fur *et al*, 1983) did not prevent the inhibitory effect of ODN, OP, and cOP on food intake. Conversely, cDLOP, which acts as a metabotropic endozepine receptor antagonist (Leprince *et al*, 2001), suppressed the anorexigenic effect of ODN, OP, and cOP. These data indicate that the effects of ODN and its analogs on feeding behavior are mediated through activation of the recently characterized metabotropic receptor. Moreover, at the highest dose tested (1000 ng/mouse), cDLOP not only suppressed the inhibitory effect of ODN on food intake, but significantly increased by its own food consumption, indicating that endogenous ligands of the metabotropic endozepine receptor, including ODN, exert a tonic inhibitory effect on feeding behavior. Of note, cDLOP has already been used successfully to decipher physiological actions of endozepines in the brain. Thus, it has been recently shown that cDLOP not only prevented the inhibitory effects of ODN on gonadotropin-releasing hormone (GnRH) and NPY gene expression but also increased by itself GnRH and NPY mRNA levels (Compère *et al*, 2004, 2005). Consistent with an endozepinergic tonus controlling feeding, in the present study, we found that the stimulatory action of cDLOP on food consumption was completely abolished by ODN. These data indicate that the orexigenic effect of cDLOP and the anorexigenic effect of ODN are mediated through activation of the same receptor. This latter observation provides strong evidence for the involvement of endozepine metabotropic receptors in the control of feeding.

In conclusion, the present study provides direct evidence that, in mice, the anorexigenic effect of ODN involves receptors that are distinct from the classical CBR and PBR. The pharmacological profile of these receptors corresponds to that of the metabotropic endozepine receptor positively coupled to phospholipase C. Our results also suggest the existence of an endozepinergic tonus that appears to play an important role in the regulation of feeding behavior.

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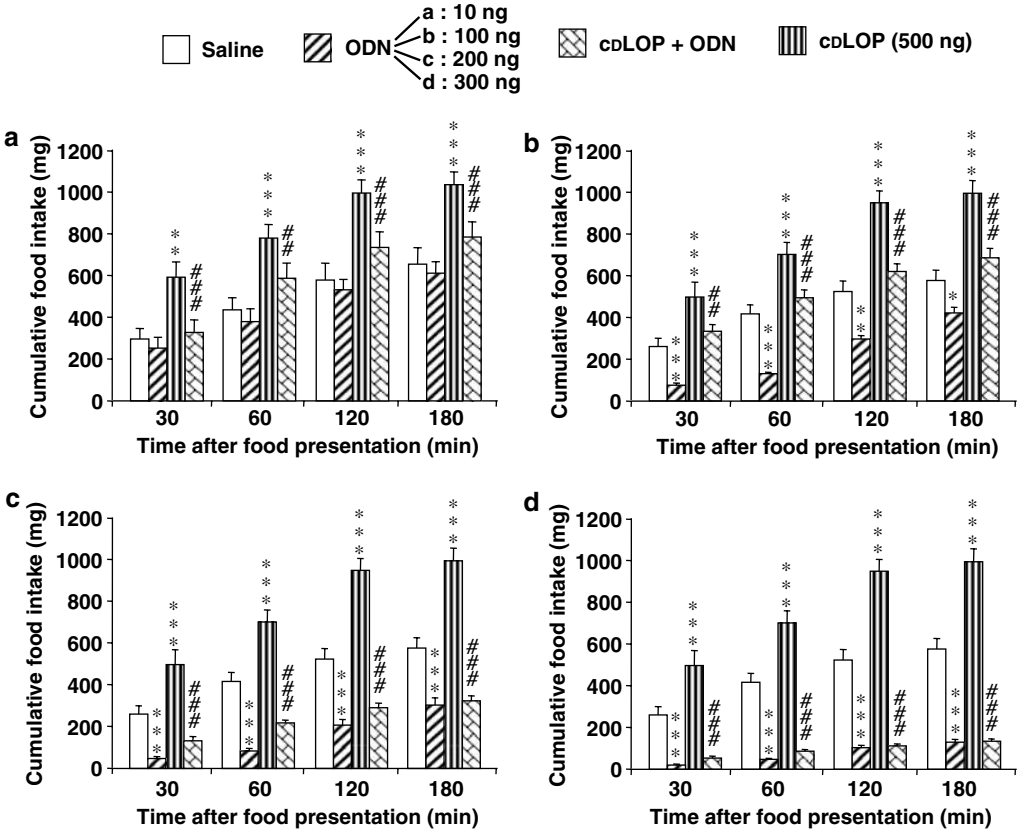

Figure 9 Effect of graded doses of ODN on cDLOP-induced increase of food intake in food-restricted mice. Mice were food restricted (half of their daily food consumption) for 18 h and then injected i.c.v. (10 µl) with saline or cyclo$_{1-8}$[D-Leu^5]OP (cDLOP; 500 ng/mouse) in the absence or presence of increasing doses of ODN (a, 10 ng/mouse; b, 100 ng/mouse; c, 200 ng/mouse; d, 300 ng/mouse). Ten min after i.c.v. injection, each animal had access to a weighed food pellet. Cumulative food intake was measured during 3 h at the time indicated. Mean ± SEM represents data from 10–20 mice per group. Student–Newman–Keuls test *post hoc*: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* saline-injected mice; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs* cDLOP-injected mice. A two-way ANOVA revealed a significant interaction between ODN and cDLOP: at 30 min $F(4,110) = 4.01$, $p < 0.01$, at 60 min $F(4,110) = 5.2$, $p < 0.001$, at 120 min $F(4,110) = 8.75$, $p < 0.001$, and at 180 min $F(4,110) = 8.13$, $p < 0.001$.

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